Customer No.: 07278 Docket No. 5986/1L712US1

EXPRESS MAIL CERTIFICATE

8/28/03 EV306630250-US

Date Label No.

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 by "Express Mail Post Office to Addressee" service.

D B Peck

Name (Print) Signature

PLEASE CHARGE ANY DEFICIENCY UP TO $300.00 OR CREDIT ANY EXCESS IN THE FEES DUE WITH THIS DOCUMENT TO OUR DEPOSIT ACCOUNT NO. 04 - 0100

METHODS OF SCREENING TEST COMPOUNDS USING GADD34L, AN eIF2α-SPECIFIC PHOSPHATASE SUBUNIT

This application claims priority to provisional U.S. application number 60/408,679 filed September 6, 2002 under 35 U.S.C. § 119(e), which is incorporated by reference.

This invention was made with Government support under Grant No. ES08681 awarded by the NIEHS and Grant No. DK47119 awarded by NIDDK. The United States Government may have certain rights to this invention pursuant to these grants.

Numerous references, including patents, patent applications, and various publications are cited and discussed in the description of this invention. The citation and/or discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the invention described herein. All references cited and discussed in this specification are incorporated herein by reference in their entirety and to the same extent as if each reference was individually incorporated by reference.

FIELD OF INVENTION

The invention is directed to methods and reagents for identifying test substances useful for the prevention or treatment of diseases involving an oxidative stress. The methods involve screening assays, including high throughput screening techniques, in

which the test substances are tested for their ability to promote resistance to oxidative stress by inhibiting the activity of GADD34L and thereby inhibiting dephosphorylation of eIF2α, in cells not subject to stress, while not causing stress.

BACKGROUND OF THE INVENTION

Primary reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide, hydroxyl radicals, and ortho-quinone derivatives of catecholamines, exert their cellular effects by modifying DNA, lipids, and proteins to form secondary electrophiles. Examples of such secondary electrophiles include hydroxyalkenals, nucleotide propenals, and hydroxyperoxy fatty acyl chains. Secondary electrophiles are implicated in cellular dysfunction either because they are no longer able to participate in normal cellular activity or because they serve as electron acceptors in oxidative chain reactions that result in the modification of other essential cellular components. Damage caused by primary and secondary ROS contributes to the pathogenesis of several acute human diseases. ROS likely participate in the central nervous system damage caused by neuronal ischemia during stroke, post-cardiopulmonary bypass syndrome, brain trauma, and status epilepticus, as well as the cardiac damage induced during ischemic heart disease and renal damage induced by ischemia and toxins. ROS also likely participate in chronic human diseases such as the destruction of the islets of Langerhans of the endocrine pancreas in Diabetes Mellitus, the destruction of neurons in Parkinson's disease, and other chronic neurodegenerative disorders.

One way that cells handle the deleterious effects of ROS is via the preconditioning response, an adaptation whereby cells are rendered resistant to injury by prior exposure to smaller doses of the same ROS-inducing stress, which threatens to cause the injury in question. Thus, an ideal therapeutic strategy for the prevention and/or treatment of diseases involving an oxidative stress would involve stimulation of the preconditioning response without causing cellular injury. However, identification of potentially useful therapeutic agents has been hampered by the fact that compounds that cause the preconditioning response generally also cause stress to the cell.

Recently, the signaling pathways involved in the cellular response to oxidative stress have been elucidated (see Figure 1). For example, the accumulation of malfolded

proteins in the endoplasmic reticulum (ER stress) leads to accumulation of ROS. ER stress activates the protein kinase PERK, which in turn phosphorylates the translation initiation factor eIF2 on its alpha subunit [Harding, H., Zhang, Y., and Ron, D. (1999). Translation and protein folding are coupled by an endoplasmic reticulum resident kinase. Nature 397, 271-274]. A different eIF2α kinase, GCN2, phosphorylates eIF2α in response to nutritional stress [Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099-1108]. eIF2α phosphorylation leads to marked reduction in protein biosynthesis [Harding, H., Zhang, Y., Bertolotti, A., Zeng, H. and Ron, D. (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol. Cell 5, 897-904] and to the expression of the transcription factor ATF4, which then activates stress response genes in a signaling pathway termed the Integrated Stress Response (ISR) [Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099-1108].

This activated Integrated Stress Response pathway is down-regulated by the activity of a phosphatase holoenzyme that dephosphorylates eIF2α on serine 51 (in yeast eIF2α, corresponding to residue 52 in rodents or humans). The phosphatase holoenzyme consists of the catalytic subunit of protein phosphatase 1 (PP1c) and GADD34, an eIF2α-specific regulatory subunit of the phosphatase [Novoa, I.; Zeng, H., Harding, H., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol., 153, 1011-1022].

The expression of Integrated Stress Response target genes promotes resistance to both the stress of malfolded proteins in the endoplasmic reticulum and to the consequences of ROS accumulation. Methods to monitor activation of the Integrated Stress Response provide for effective screens to identify test substances capable of promoting preconditioning by activating the pathway in cells not subject to stress. Specifically, these screening methods to assess activation of the ISR provide the advantage of the identification of test substances, which activate the pathway, yet do not cause cell stress.

Substances that inhibit the eIF2α-specific phosphatase could promote the accumulation of phosphorylated eIF2α in cells not subject to stress, and thereby activate a protective ISR response without subjecting the cell to stress. Such substances would therefore be useful in the prevention and/or treatment of diseases involving an oxidative stress. GADD34 is limited in its use as a therapeutic target in this scheme as it is only expressed following stress-induced activation of eIF2α kinases, and not in cells not subject to stress. Therefore, inhibition of GADD34 is unlikely to provide a protective ISR response against oxidative stress in cells not subject to stress..

This invention involves the discovery of a GADD34-related regulatory subunit of PP1c, hereinafter referred to as GADD34-Like or GADD34L. Inhibition of GADD34L activity in cells not subject to stress leads to increased phosphorylation of eIF2α and to activation of the ISR. Therefore, GADD34L represents a useful therapeutic target for the promotion of preconditioning to prevent and/or treat diseases involving an oxidative stress. Furthermore, screening for test substances that are inhibitors of GADD34L activity provides the advantage of identifying therapeutic agents to prevent and/or treat diseases involving oxidative stress by activation of the ISR pathway, that do not provide cells stress.

SUMMARY OF INVENTION

This invention is thus directed to a method for screening a plurality of test substances useful for the prevention or treatment of a disease involving an oxidative stress, which comprises the steps of i) testing each of the test substances for its ability to inhibit the activity of GADD34L, and ii) identifying the test substance which inhibits the activity of GADD34L, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. This invention is further directed to a method for identifying a test substance useful for the prevention or treatment of a disease involving an oxidative stress, which comprises testing a test substance for its ability to inhibit the activity of GADD34L, thereby to determine whether the substance promotes resistance to cell stress, and to identify said substance as a preventive or therapeutic agent for a disease involving an oxidative stress.

In one embodiment, the test substance promotes preconditioning and resistance to injury due to oxidative stress by inhibiting activity of the GADD34L protein. In a further embodiment, the test substance inhibits the association of GADD34L protein with the phosphatase PP1c. In another embodiment, the test substance inhibits the translation of GADD34L mRNA. In another embodiment, the test substance inhibits the transcription of the GADD34L genomic locus. In another embodiment, the method further comprises a step of verifying whether said test substance does not cause stress to cells. In a further embodiment, due to its effects on GADD34L, the test substance causes an accumulation of phosphorylated eIF2α in cells not subject to stress. In a further embodiment, due to its effects on GADD34L, the test substance inhibits dephosphorylation of eIF2α. In a further embodiment, due to its effects on GADD34L, the test substance activates the expression of Integrated Stress Response target genes. In a further embodiment, due to its effects on GADD34L, the test substance promotes cell survival following exposure to toxic concentrations of an agent which induces oxidative stress.

The invention is directed to a method for the prevention or treatment of a disease involving an oxidative stress in a patient in need of such treatment, which comprises administering to the patient an effective amount of a GADD34L phosphatase inhibitor identified for its ability to promote resistance to cell stress while not causing stress.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1: A schematic description of a portion of the Integrated Stress Response (ISR). Kinases such as PERK and GCN2 respond to a variety of stresses by establishing a signaling pathway that converges on eIF2α phosphorylation. Phosphorylation of eIF2 on its α subunit represses translation of most mRNAs, while enhancing translation of special mRNAs such as ATF4. ATF4, a transcription factor, activates expression of various ISR target genes, including CHOP and GADD34. GADD34 encodes an eIF2α-specific regulatory subunit of the PP1c holophosphatase complex that terminates signaling in the ISR by dephosphorylating eIF2α.

FIGURE 2A and 2B: Full length human GADD34-Like (GADD34L) cDNA sequence (SEQ ID NO: 1). The open reading frame encoding the full length human GADD34L protein is encompassed by nucleotides 407 through 2548, inclusive. The start codon for the GADD34L protein is bold and underlined (**ATG, FIGURE 2A**), while the stop codon is underlined (TGA, **FIGURE 2B**). This sequence is identical to that of Genbank Accession Numbers NM_032833 and AK027650.

FIGURE 3: Full length human GADD34-Like (GADD34L) amino acid sequence (SEQ ID NO: 2). This protein sequence is encoded by nucleotides 407 through 2545, inclusive, of the full length human GADD34L cDNA sequence (see Figure 2A and 2B). This sequence is identical to that of Genbank Accession Number NM_032833.

FIGURE 4A, 4B, 4C, and 4D: Full length mouse GADD34-Like (GADD34L) cDNA sequence (SEQ ID NO: 3). The open reading frame encoding the full length mouse GADD34L protein is encompassed by nucleotides 462 through 2558, inclusive. The start codon for the GADD34L protein is bold and underlined (**ATG**, **FIGURE 4A**), while the stop codon is underlined (TGA, **FIGURE 4B**).

FIGURE 5: Full length mouse GADD34-Like (GADD34L) amino acid sequence (SEQ ID NO: 4). This protein sequence is encoded by nucleotides 462 through 2555, inclusive, of the full length mouse GADD34L cDNA sequence (see Figure 4A, 4B, 4C and 4D).

FIGURE 6: Inhibition of endogenous GADD34L protects cells against oxidative toxicity. The bar graphs show percent survival of HT22 cells following exposure to toxic amounts of glutamate. HT22 cells were treated with 70μM 22P19 for 24 or 48h (top panel), or left untransfected (NTx), mock transfected, transfected with GADD34L siRNA, or transfected with control or CD2 siRNA (bottom panel), and then exposed to the indicated concentrations of glutamate for 18 hours. 100% survival is defined as the level of MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma) cleavage in cells that had not been exposed to glutamate in each treatment group. Shown are the means ± SEM of a representative experiment performed in duplicate and repeated

four times. There was dramatically improved cell survival following pre-treatment with 22P19, or RNAi-based inhibition of GADD34L. 22P19 is a chemical inhibitor of GADD34L isolated in a high-throughput screen based on its ability to protect cells from tunicamycin toxicity.

FIGURE 7: The structure of 22P19. The chemical compound called 22P19 was isolated by screening the Chembridge™ library (Chembridge San Diego, CA) for compounds that protect PC-12 cells from death induced by prolonged exposure to tunicamycin.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner in describing the devices and methods of the invention and how to make and use them. For convenience, certain terms are highlighted, for example using italics and/or quotation marks. The use of highlighting has no influence on the scope and meaning of a term; the scope and meaning of a term is the same, in the same context, whether or not it is highlighted. It will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification, including examples of any terms discussed herein, is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to the preferred embodiments.

Primary reactive oxygen species (ROS) include, but are not limited to, superoxide radical, hydrogen peroxide, hydroxyl radical, and ortho-quinone derivatives of

catecholamines. Primary ROS exert their cellular effects by modifying DNA, lipids and proteins to form secondary electrophiles. The secondary electrophiles are also implicated in cellular dysfunction either because they are no longer able to participate in normal cellular activity or because they serve as electron acceptors in oxidative chain reactions that result in the modification of other essential cellular components. Examples of such latter *secondary electrophiles* or *secondary reactive oxygen species* include hydroxyalkenals, nucleotide propenals, and hydroxyperoxy fatty acyl chains.

Oxidative stress or *stressful conditions* involves any actions by primary or secondary reactive oxygen species on the body.

Cell stress includes oxidative stress, ER stress, and nutritional stress on the cell and any subsequent cell injury due to the initial oxidative stress, ER stress, and nutritional stress.

Diseases involving an oxidative stress have a pathogenesis related to the damage caused by the primary and secondary ROS. ROS contribute to the pathogenesis of important human diseases caused by neuronal ischemia during stroke, post-cardiopulmonary bypass syndrome, brain trauma, and status epilepticus. ROS likely participate in cardiac damage induced during ischemic heart disease, renal damage induced by ischemia and toxins as well as in chronic diseases such as the destruction of neurons in Parkinson's disease, Amyloidoses, Prion disorders, Alzheimer's disease, and other chronic neurodegenerative disorders. Autoimmune diseases such as the destruction of the islets of Langerhans of the endocrine pancreas in Diabetes Mellitus are also encompassed.

Preconditioning is the effect in which a low dose of a stressful stimulus associated with oxidative stress promotes resistance to ROS. This effect is a natural cellular defense strategy to combat the effects of ROS.

Target gene and *target protein* are understood to refer to the gene or protein of the Integrated Stress Response pathway whose activation or inhibition is determined in the screening methods of the invention. The target genes or proteins are meant to refer to genes or proteins of any origin, regardless of the species. Substantially all the target genes or proteins used in the methods of the invention can be obtained from higher eukaryote organisms, such as mammalian or bird genes or proteins. They may more particularly be

rodent or primate genes or proteins, preferably human. However certain of the genes or proteins used in the methods of the invention may alternatively be obtained from lower organisms such as yeasts. They may have homologous wild-type sequences or be function-conservative variants. *Function-conservative variants* are those in which a given amino acid residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Such changes are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. Amino acids other than those indicated as conserved may differ in a protein so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A function-conservative variant also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, most preferably at least 85%, and even more preferably at least 90%, and which has the same or substantially similar properties or functions as the native or parent protein or enzyme to which it is compared.

As used herein, the term *homologous* in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," and homologous proteins from different species. Such proteins (and their encoding genes) have sequence homology, as reflected by their sequence similarity, whether in terms of percent similarity or the presence of specific residues or motifs at conserved positions. Accordingly, the term *sequence similarity* in all its grammatical forms refers to the degree of identity or correspondence between nucleic acid or amino acid sequences of proteins that may or may not share a common evolutionary origin. In a specific embodiment, two DNA sequences are *substantially homologous* or *substantially*

similar when at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences, as determined by sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, etc. An example of such a sequence is an allelic or species variant of the specific genes of the invention. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Similarly, in a particular embodiment, two amino acid sequences are substantially homologous or substantially similar when greater than 80% of the amino acids are identical, or greater than about 90% are similar (functionally identical). Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 7*, Madison, Wisconsin) pileup program, or any of the programs described above (BLAST, FASTA, etc.).

A nucleic acid molecule is *hybridizable* to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (*see* Sambrook *et al.*, 1989 *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m (melting temperature) of 55°C, can be used, *e.g.*, 5x SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5x SSC, 0.5% SDS). Moderate stringency hybridization conditions correspond to a higher T_m, *e.g.*, 40% formamide, with 5x or 6x SCC. High stringency hybridization conditions correspond to the highest T_m, *e.g.*, 50% formamide, 5x or 6x SCC. SCC is a 0.15M NaCl, 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of

nucleic acids having those sequences. In a specific embodiment, the term *standard hybridization conditions* refers to a T_m of 55°C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60°C; in a more preferred embodiment, the T_m is 65°C. In a specific embodiment, "high stringency" refers to hybridization and/or washing conditions at 68°C in 0.2XSSC, at 42°C in 50% formamide, 4XSSC, or under conditions that afford levels of hybridization equivalent to those observed under either of these two conditions.

Sequence-conservative variants are gene variants in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position.

Expression of a gene is understood to include both transcription and/or translation events.

A *coding sequence* or a sequence *encoding* an expression product, such as an RNA, polypeptide, protein or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein or enzyme; i.e., the nucleotide sequence *encodes* that RNA or it encodes the amino acid sequence for that polypeptide, protein or enzyme.

A *promoter sequence* is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently found, for example, by mapping with nuclease S1), as well as consensus sequences for protein binding responsible for the binding of RNA polymerase.

A coding sequence is *under the control of* or is *operatively associated with* transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into RNA, which is then spliced (if it contains introns) and, if the sequence encodes a protein, is translated into that protein.

The term *transfection* means the introduction of a "foreign" (i.e., extrinsic or extracellular) gene, DNA or RNA sequence into a eukaryotic host cell. The term *transformation* means the introduction of a "foreign" (i.e., extrinsic or extracellular) gene, DNA or RNA sequence into a prokaryotic host cell. In the case of both *transfection* and *transformation* the host cell will express the introduced gene or sequence to produce a desired substance, in this invention typically an RNA coded by the introduced gene or sequence, but also a protein or an enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences (e.g., start, stop, promoter, signal, secretion or other sequences used by a cell's genetic machinery). The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been *transformed* and is a *transformant* or a *clone*. The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell or cells of a different genus or species.

The terms *vector*, *cloning vector* and *expression vector* mean the vehicle by which a DNA or RNA sequence (e.g., a foreign gene) can be introduced into a host cell so as to transform the host and promote expression (e.g., transcription and translation) of the introduced sequence. Vectors may include plasmids, phages, viruses, etc. and are discussed in greater detail below.

A *cassette* refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily introduced into a suitable host cell. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of

eukaryotic and prokaryotic hosts. The term *host cell* means any cell of any organism that is selected, modified, transformed, grown or used or manipulated in any way for the production of a substance by the cell. For example, a host cell may be one that is manipulated to express a particular gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays that are described infra. Host cells may be cultured in vitro or one or more cells in a non-human animal (e.g., a transgenic animal or a transiently transfected animal).

The term *expression system* means a host cell and compatible vector under suitable conditions, e.g. for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Common expression systems include E. coli host cells and plasmid vectors, insect host cells such as Sf9, Hi5 or S2 cells and Baculovirus vectors, Drosophila cells (Schneider cells) and expression systems, and mammalian host cells and vectors.

The term *heterologous* refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is a such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, a gene encoding a protein of interest, e.g., a GADD34L gene, is heterologous to the vector DNA in which it is inserted for cloning or expression, and it is heterologous to a host cell containing such a vector, in which it is expressed, e.g., a CHO cell. with a different gene that the one it is operatively associated with in nature.

The phrase *cells that overexpress GADD34L, or portions thereof,* is taken to indicate host cells of any type which have been transformed with an expression vector driving expression of exogenous GADD34L protein. The GADD34L protein expressed may represent a partial or complete GADD34L polypeptide as encoded by partial or complete GADD34L nucleic acid sequences within the expression vector. The GADD34L protein and nucleic acid sequences may be of mouse, human, hamster, or other mammalian origin. The GADD34L encoding sequences used may comprise function-conservative variants or sequence conservative variants of GADD34L. For use

in the claimed methods, partial GADD34L polypeptides thus expressed must maintain partial or complete GADD34L activity as a suppressor of the Integrated Stress Response. Partial GADD34L polypeptides already defined which maintain such activity include: the C-terminal hamster GADD34L peptide encoded by the retroviral clone CD-GSE; the C-terminal mouse GADD34L peptide, comprising amino acids 418-698 of the full length protein, such as encoded in the expression construct pCMV2FLAG-ΔN GADD34L; and the N-terminally truncated mouse GADD34L peptide, comprising amino acids 24-698 of the full length protein, such as encoded in the expression construct pCMV2FLAG FL GADD34L.

Test substance or *test compound* is a chemically defined compound or mixture of compounds (as in the case of a natural extract or tissue culture supernatant) whose ability to promote resistance to cell stress, while not causing stress, is defined by the assays of the invention. These compounds or mixtures of compounds include, but are not limited to, chemicals including chemical libraries, peptides including peptide libraries, cDNA libraries such as retroviral cDNA libraries, phage display libraries, cell culture supernatants, cell extracts, and cell lysates.

The term *therapeutically effective dose* refers to that amount of a compound or compositions that is sufficient to result in a desired activity.

The phrase *pharmaceutically acceptable* refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar adverse reaction (for example, gastric upset, dizziness and the like) when administered to an individual. Preferably, and particularly where a vaccine is used in humans, the term "pharmaceutically acceptable" may mean approved by a regulatory agency (for example, the U.S. Food and Drug Administration) or listed in a generally recognized pharmacopeia for use in animals (for example, the U.S. Pharmacopeia).

The term *carrier* refers to a diluent, adjuvant, excipient, or vehicle with which a compound is administered. Sterile water or aqueous saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Exemplary suitable pharmaceutical carriers are described in "Reminington's Pharmaceutical Sciences" by E.W. Martin.

The terms *about* and *approximately* shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typical, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Alternatively, and particularly in biological systems, the terms "about" and "approximately" may mean values that are within an order of magnitude, preferably within 5-fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term "about" or "approximately" can be inferred when not expressly stated.

General Methods

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (referred to herein as "Sambrook et al., 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins, eds. 1984); Animal Cell Culture (R.I. Freshney, ed. 1986); Immobilized Cells and Enzymes (IRL Press, 1986); B.E. Perbal, A Practical Guide to Molecular Cloning (1984); F.M. Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994). Conventional techniques for *in vitro* cell culture and treatment, lysate preparation, immunoprecipitation, Western blotting, and immunodetection are also described in the art See for example, Harding, H., Zhang, Y., and Ron, D. (1999). Translation and protein folding are coupled by an endoplasmic reticulum resident kinase. Nature 397:271-274; Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell. 6:1099-1108; Harding, H., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. Mol. Cell 5:897-904; and Novoa, I.; Zeng, H., Harding, H., and Ron, D. (2001).

Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol., 153, 1011-1022.

The screening methods related to the Integrated Stress Response pathway are directed to different activation sites of the pathway, which includes, but is not limited to, the components illustrated in Figure 1. The methods include:

1) Activated expression of Integrated Stress Response target genes included in Tables 1 and 2, ATF4, also known as CREB2, TAXREB67, and C/ATF4 (Harding, H., Novoa, I., Zhang, Y., Zeng, H., Shapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099-1108.), and of any target genes that can be identified by cDNA expression microarrays, that results from inhibition of the activity of GADD34L.
2) Increased phosphorylation of eIF2α [Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099-1108], caused by inhibition of the activity of GADD34L.
3) Inhibition of dephosphorylation of phosphorylated eIF2α. caused by inhibition of the activity of GADD34L.

These various endpoints can be measured by anyone skilled in the art of cell biology using the following methods.

Activation of expression of any target genes or proteins can be assessed by determining either the level of transcription or the level of translation, in the presence of test substance, in comparison with control assays performed in the absence of the test substance. Such assays may be performed on cells capable of expressing the target gene or a surrogate thereof, such as a reporter gene. The levels of phosphorylation of target proteins can be assessed by various methods, including immunoassays or radiolabeling.

Table 1: Target genes of the Integrated Stress Response identified by cDNA expression microarrays and Northern blot analysis

Gene Name	Accession number (GenBank)
GLYT1	W90900
Cystathionine gamma-lyase	AA096870
Methylenetetrahydrofolate dehydrogenase	W84014
Serine Hydroxymethyltransferase	AA208877
Heme Oxigenase-1	AA213167
XCTc	AA049696
Coproporphyrinogen oxidase	AA259342
CHOP	NM007837
GADD34	AA0050417

Table 2: Target genes of the Integrated Stress Response (Genes with statistically significant reduced ER stress inducibility in PERK mutant cells)

Gene Name(s)	Product/homology	Putative/known functional category	Accession number (GenBank)
Tj6	vacuolar ATPase, proton pump homologue	secreted pathway function	AA881202
Sec23b	homologue of Sec23b SEC23B (S. cerevisiae)	secreted pathway function	AI848343
Ugalt, Had1	UDP-galactose translocator 2	secreted pathway function	D87990
1500026A19Rik	dolichyl-phosphate beta-glucosyltransferase homologue	secreted pathway function	AA111463
Gpnat1	Glucosamine-phosphate N-acetyltransferase 1	secreted pathway function	AW123026
Pig-a	GPI-anchor biosynthesis (PIG-A protein)	secreted pathway function	D31863
Sel1h	Sel1 (suppressor of lin-12) 1 homolog (C. elegans)	secreted pathway function	AF063095
Sel1l	negative regulator of Notch, promotes ERAD	secreted pathway function	AW121840

WRN typeII	Werner syndrome homologue; helicase	stress response	D86527
A170, STAP	oxidative stress inducible	stress response	U40930
p58, Prkri, mp58	interferon inducible PKR inhibitor, DnaJ (Hsp40)	stress response	U28423
p58, Dnajc3	interferon inducible PKR inhibitor, DnaJ (Hsp40)	stress response	U28423
Dnajb9	DnaJ (Hsp40) homologue, subfamily B, member 9	stress response	AW120711
mATF4	Activating transcription factor 4	transcription/stress response	M94087
LRG-21, ATF3	Activating transcription factor 3	transcription/stress response	U19118
EST1	alanine tRNA synthetase homologue	translation or amino acid metabolism	AI839392
Wars	Tryptophanyl-tRNA synthetase	translation or amino acid metabolism	AI851163
1110068E11Rik	translation initiation factor eIF-4A -homologue	translation or amino acid metabolism	AW124530
Rnu22 RNA	RNA, U22 small nucleolar	Ribosome biogenesis	AA684508
GU2	Nucleolar protein GU2, probable RNA helicase	Ribosome biogenesis	AA866971
Snk	Serum-inducible kinase	signaling	M96163
Fyn	proto-oncogene, tyrosine protein kinase	signaling	M27266
5730434I03Rik	BTF3 homologue (basal transcription factor)	transcription	AI846097
Mpc2, Cbx4	Chromobox homologue 4; transcriptional repressor	transcription	U63387
Ets-2	E26 avian leukemia oncogene 2, 3' domain	transcription	J04103
c-myc	c-myc	transcription	L00039
Arnt3, Bmal1	CLOCK and NPAS2 dimer partner, regulated by NADH	transcription	AB014494
E4BP4	NFIL3/E4BP4 transcription, circadian rhythm regulated	transcription	U83148
Etv6	Ets variant gene 6 (TEL oncogene)	transcription	AI845538
2310004B05Rik	group XII secreted phospholipase A2	secreted protein	AI845798
EST2	similar to extracellular matrix protein trichohyalin	secreted protein	AA612483
Actb	Actb Actin, beta, cytoplasmic	cytoskelatin	M12481
Ghitm	Growth hormone inducible transmembrane protein	growth/differen-tiation	AW120976
EST3	UCP2 mitochondrial uncoupling protein homologue	mitochondrial function	AW125634

Rnu1a-1	Small nuclear ribonucleoprotein polypeptide A	RNA/DNA housekeeping	L15447

Host cells

A broad variety of host-expression vector systems may be utilized to express the coding sequences of the proteins used in the assays of this invention. These include, but are not limited to, mammalian cell systems such as Cos-7, CHO, BHK, 3T3, HEK293, HT22, and 293T cells. The mammalian cell systems may harbor recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter or the vaccine virus 7.5K promoter).

Additional host-expression vector systems include, but are not limited to, microorganisms such as bacteria (e.g., E.coli or B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing PTK or adaptor protein coding sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the protein or peptide coding sequences; insect cell systems, such as Sf9 or Sf21 infected with recombinant virus expression vectors (e.g., baculovirus) containing the protein or peptide coding sequences; amphibian cells, such as Xenopus oocytes; or plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the protein or peptide coding sequence. Culture conditions for each of these cell types is specific and is known to those familiar with the art.

In one example, COS-7 cells are grown on 150 mm plates in DMEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37 °C., 5% CO_2. Stock plates of COS-7 cells are trypsinized and split 1:6 every 3-4 days.

In another example, CHO cells are grown on 150 mm plates in HAM's F-12 medium with supplements (10% bovine calf serum, 4 mM L-glutamine and 100 units/ml penicillin/100 μg/ml streptomycin) at 37 °C., 5% CO_2. Stock plates of CHO cells are trypsinized and split 1:8 every 3-4 days.

DNA encoding proteins to be assayed can be transiently or stably expressed in the cell lines by several methods known in the art, such as calcium phosphate-mediated, DEAE-dextran mediated, liposomal-mediated, viral-mediated, electroporation-mediated and microinjection delivery. Each of these methods may require optimization of assorted experimental parameters depending on the DNA, cell line, and the type of assay to be subsequently employed. In one embodiment, GADD34L protein, or portions thereof, may be expressed in cell lines using one or more of these methods.

In addition, native cell lines that naturally carry and express the nucleic acid sequences for the target protein may be used.

Activation of target genes

The screening of test substances may be assessed by determining either the level of transcription of the target genes or the level of translation of the target proteins encoded by the genes, in the presence of the test substance. These target genes are herein identified as genes whose expression is modified in response to an oxidative stress. They include the target genes of Table 1. The assays may be performed on cells capable of expressing the target gene or a surrogate thereof, such as a reporter gene.

Reporter gene assays of the invention may use one or more of the commonly used detection techniques involving isotopic, colorimetric, fluorimetric, or luminescent enzyme substrates and immuno-assay based procedures with isotopic, colorimetric, or chemiluminescent end points. The assays of the invention include, but are not limited to, using the reporter genes for the following proteins: CAT (chloramphenicol acetyltransferase), which transfers radioactive acetyl groups to chloramphenicol for detection by thin layer chromatography and autoradiography; GAL (β-galactosidase), which hydrolyzes colorless galactosides to yield colored products; GUS (β-glucuronidase), which hydrolyzes colorless glucuronides to yield colored product; LUC (luciferase), which oxidizes luciferin emitting photons; GFP (green fluorescent protein), which fluoresces on irradiation with UV; and hGH (human growth hormone), which is detected using a radioimmunoassay, and SEAP (a secreted form of the human placental alkaline phosphatase), which is detected with both colorimetric and chemiluminescent substrates.

Assays to monitor transcription of the target gene or the surrogate gene may be carried out by means of a Northern blot. Assays to monitor translation of the target gene or the surrogate gene may be carried out either by an immunoassay described herein or by utilizing the various read-outs for surrogate reporter genes described herein.

In one example, test substances, which inhibit the activity of GADD34L and in turn activate *CHOP* are assayed with a surrogate reporter gene. Chinese Hamster Ovary cells (CHO) are stably transfected with the GFP reporter gene fused to the *CHOP* gene to form a CHOP::GFP CHO cell line (Wang, X. Z., Harding, H. P., Zhang, Y., Jolicoeur, E. M., Kuroda, M., and Ron, D. (1998). Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J. 17, 5708-5717). This cell line may be treated with test compounds and the activity of the marker gene, GFP, may be monitored to identify compounds, which activate the Integrated Stress Response. In another embodiment, by substituting a LUC or GAL reporter for the GFP reporter, stably transfected CHO cells may be adapted for use in high throughput screening of libraries of compounds.

In another example, translation of CHOP may be detected immunochemically in cultured cells exposed to test substances, by immunoblot or by immunocytochemistry described in the immunoassays herein, with antisera to CHOP as described in [Wang, X.-Z., Lawson, B., Brewer, J., Zinszner, H., Sanjay, A., Mi, L., Boorstein, R., Kreibich, G., Hendershot, L., and Ron, D. (1996). Signals from the stressed endoplasmic reticulum induce C/EBP homologous protein (CHOP/GADD153). Mol. Cell. Biol. 16:4273-4280]. The antisera to CHOP may also be adapted to an ELISA-based assay for measuring CHOP expression, allowing high throughput screening for compounds that promote CHOP translation.

In another embodiment, ATF4 translation may be detected by a surrogate assay using a stable cell line containing a reporter gene, such as LUC, controlled by the translational regulatory elements of the ATF4 mRNA (Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099-1108). The reporter gene is linked to all or part of the ATF4 promoter, in particular the ATF4 gene's translational regulatory sequences.

General Immunoassays

Various assays utilizing binding partners are useful in the screening methods of the invention. Preferably such binding partners are antibodies and the assays are called immunoassays. The below description refers to the use of antibodies, but it is understood that any other binding partner may be useful as well. Immunoassays are techniques known in the art, and include, for example, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, immunodiffusion assays, in situ immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, immunofluorescence assays, and immunoelectrophoresis assays.

Monoclonal antibodies or polyclonal antibodies selective for the target protein are selected by techniques well known in the art. Immunoblots can be performed using lysates from cells that express the target protein to determine specificity. The preferred antibody will only bind to the target protein, preferably greater than 100,000 molecules per cell. An alternative method for determining specificity is immunoprecipitation. The binding affinity of the monoclonal antibody or polyclonal antibody for the substance can be determined by the relative strength of the signal generated in the immunoblot or by other techniques well known in the art.

A known number of cells expressing the target protein is lysed and serial dilutions of the lysate are applied to wells in a 96 well microtiter plate that have been precoated with the anchoring antibody. After allowing the substance to bind to the antibody, the unbound material is washed away and the amount of bound substance is determined using known immunoassay techniques. In order to have the proper signal to noise ratio one must be able to detect the target molecule in at least $1x10^4$ cell equivalents per well. The maximum number of cells allowable per well is generally $<1x10^5$ due to space constraints although this number may be somewhat larger or smaller depending on the cell type. The antibodies used in the immunoassays of the invention include, but are not limited, to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

For the production of antibodies, various host animals, including but not limited to rabbits, mice, rats may be immunized by injection with the particular antigen in a

suitable adjuvant or by injecting the epitope conjugated to an immunogenic carrier. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Monoclonal antibodies may be prepared by using any technique, which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein, (Nature, 1975, 256:495-497), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72) and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Human antibodies may be used and can be obtained by using human hybridomas (Cote at al., 1983, Proc. Natl. Acad. Sci. 80:2026-2030) or by transforming human B cells with EBV virus in vitro (Cole et al., 1985, in, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). Techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

Techniques for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can also be used to produce substance-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to antigens. Antibody fragments which contain binding sites specific for the protein of interest may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')2 fragments which can be produced by pepsin

digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments.

The antibodies may be stored and purified using methods which are well known to those skilled in the art (e.g., see "Antibodies, A Laboratory-Manual", eds. Harlow & Lane, Cold Spring Harbor Laboratory, 1988, Ch. 8). Alternatively, polyclonal or monoclonal antibodies specific for the target protein may be obtained from commercial sources.

In the various immunoassays of the invention, antibody binding may be detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

The immunoassays of the invention may be carried out using an immobilized phase. Solid phases used for the immobilization of a protein may be prepared by coating with the specific antibody. In the case where a polyclonal antibody is used, the solid phase may first be coated with an anti-Ig that binds to the polyclonal antibody and indirectly immobilizes it to the solid phase. The solid phase may comprise a microtiter plate, a stick, tube, disc, fiber or the like, or a microtiter plate. A preferred solid phase is a 96 well microtiter plate such as those available from Corning, Cynatech, and Nunc. Particularly preferred 96 well plates are the Corning, Nunc MaxiSop, and Dynatech Immulon I and IV. Ideal conditions for maximum coating can vary with pH, ionic strength, and antibody concentration. Preferred conditions will be pH 6-9.5, 0-200 mM NaCl, and 1-10 μg/ml of antibody. Generally 150 μl per well is used. The antibody may be attached to the solid phase by any of a variety of methods known to those skilled in the art, including but not limited to non-covalent and covalent attachments.

The antibody may be labeled directly or can be detected using a secondary reagent. Such signal generating systems include, but are not limited to, enzyme-linked systems (such as horseradish peroxidase or alkaline phosphatase), radiolabels, fluorescent labels, light-emitting labels, light-absorbing labels, dyes, or biotin-avidin labeling

systems (e.g., See "Antibodies, A Laboratory Manual, eds. Harlow & Lane, Cold Spring Harbor Laboratory 1988, Ch. 9).

In the case of conjugated enzymes, an appropriate substrate, such as a colorimetric substrate, is added. Specific substrates used for detection include ABTS (horseradish peroxidase), DAB, AEC, BCIP/NPT (alkaline phosphatase) and BCIG (beta-galactosidase). The binding of the enzyme-conjugated anti-IgG can be then detected quantitatively by techniques well known in the art.

Measure of phosphorylation of eIF2α and the activation of kinases

The levels of phosphorylation of target proteins can be assessed by various methods, including immunoassays or radiolabeling. Specifically, the increase of phosphorylation of eIF2α may be measured, activation of the kinases that promote eIF2α phosphorylation may be assayed, and inhibition of dephosphorylation of phosphorylated eIF2α may also be determined by these techniques.

In a preferred embodiment, the level of phosphorylation of a protein is assessed by utilizing a binding partner, which should be highly specific for the target protein. It is preferred that the binding partner be an antibody. It is preferably generated against a unique epitope of the substrate. In an alternative, the binding partner should be specific for the phosphorylated form of the target protein. The detection procedure used to assess the phosphorylation state of eIF2α may for instance employ an anti-phosphoserine antibody or a peptide that recognizes and binds to phosphorylated serines. The detection antibody is preferably a polyclonal antibody to maximize the signal, but may also be specific monoclonal antibodies which have been optimized for signal generation.

In one example, levels of eIF2α phosphorylated on serine 51 (in yeast eIF2α, corresponding to residue 52 in rodents or humans) can be measured by immunoblot or immunocytochemistry utilizing a commercially available antibodies, for example, product #9721 from Cell Signalling Technology. In one embodiment, the commercially available antisera to phosphorylated eIF2α may be used to develop high throughput screening assays for test substances that promote the accumulation of phosphorylated eIF2α.

In another example, inhibition of dephosphorylation of eIF2α on serine 51 (in yeast eIF2α, corresponding to residue 52 in rodents or humans) may be assayed by screening a test substance's ability to inhibit the activity of the PP1c and GADD34L holophosphatase complex. The PP1c and GADD34L complex is active *in vitro*, and its activity may be reconstituted using recombinant proteins. A cell-free assay may be used with the PP1c/GADD34L complex in combination with phosphorylated eIF2α and test substances. By utilizing an ELISA assay, dephosphorylation of eIF2α by the PP1c/GADD34L complex, and inhibition of this dephosphorylation by a test substance, may be monitored by measuring the decrease in phosphorylated eIF2α signal.

In a further example, activation of the eIF2α kinases, PERK, GCN2, HRI, and PKR, may be measured. Activation of the kinases is associated with an autophosphorylation event on known residues in the kinase (e.g., threonine 898 of mouse GCN2 and threonine 980 of mouse PERK). By using antisera, which recognize the phosphorylated and activated forms of the kinases, activation of the kinases may be detected using immunoblot or immunochemistry, such as with an ELISA. Antisera for the phosphorylated forms of the kinases PERK and GCN2 have been developed. (Harding, H., Novoa, I., Zhang, Y., Zeng, H., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol. Cell 6, 1099-1108).

Alternatively, immunoassays may be replaced by the detection of radiolabeled phosphate according to a standard technique. This involves incubating cells with the test substances and radiolabeled phosphate, lysing the cells, separating cellular protein components of the lysate using as SDS-polyacrylamide gel (SDS-PAGE) technique, in either one or two dimensions, and detecting the presence of phosphorylated proteins by exposing X-ray film.

The phosphorylation of a protein may also be conveniently detected by migration on an electrophoresis gel and Western blot, to thereby observe whether a shift of the molecular weight of the protein occurs, a phosphorylated protein being heavier than the corresponding non-phosphorylated form.

Assays to exclude test substances that cause stress

The above assays may be utilized to establish the site of action of the test substances. However, additional steps of verifying whether the test substances do not cause stress to the cells can be contemplated. For that purpose, one can measure the level of activation of other signaling proteins activated by ER stress, but not involved in the preconditioning pathway. More particularly, one can measure the level of phosphorylation of IRE1 (Bertolotti, A., Zhang, Y., Hendershot, L., Harding, H., and Ron, D. (2000). Dynamic interaction of BiP and the ER stress transducers in the unfolded protein response. Nature Cell Biology *2*, 326-332; Harding, H., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000). *Perk* is essential for translational regulation and cell survival during the unfolded protein response. Mol Cell *5*, 897-904), utilizing any standard method such as an immunoassay using an antibody specific for the phosphorylated form of the protein. One can also measure the cleavage of the protein called ATF6 (Wang, Y. et al., J. Biol. Chem., 2000, 275(35), 27013-27020), for example by tracking the appearance of the cleaved shorter forms of the protein on a Western blot.

In the case wherein the test substance causes ER stress. This may be assayed by measuring the level of activation of other signaling proteins activated by ER stress, as mentioned herein, or by measuring the level of expression of BiP. Specifically, either the level of transcription or the level of translation of this chaperone protein is assessed. An increase in the level of expression of BiP is indicative of a stress, and therefore the test substances that promote such increases may be discarded as undesirable.

One may also assess whether the test substances promote the uncharging of tRNAs, another undesirable consequence of cell stress. Established methods may be used (Dudek, S. M. et al., J. Biol. Chem., 1995, 270(49), 29323-29329). Specifically, the assay involves isolating tRNA, a fraction of which is charged with an amino acid and a fraction of which is not. One aliquot is oxidized, which prevents any initially uncharged tRNA from subsequently being acylated with an amino acid. Oxidation does not affect the ability of initially charged tRNA to be reacylated in vitro after the attached amino acid is removed, because the presence of the amino acid protects the 3' terminus of the tRNA from damage by periodate. A second aliquot is left unoxidized, allowing the determination of the total charging capacity of tRNA. The percentage of charged tRNA

is determined by dividing the counts from in vitro charging reactions (using radiolabeled amino acids) using oxidized samples by the counts from reactions using unoxidized samples.

High throughput screening

The above assays may be performed using high throughput screening techniques for identifying test substances for developing drugs that, when added to cells, promote resistance to ROS without causing stress themselves. High throughput screening techniques may be carried out using multi-well plates (e.g., 96-, 389-, or 1536- well plates), in order to carry out multiple assays using an automated robotic system. Thus, large libraries of test substances may be assayed in a highly efficient manner.

A preferred strategy for identifying test substances starts with cultured cells that overexpress GADD34L, or portions thereof, and are transfected with a reporter gene fused to the promoter of any gene that is activated by the stress response pathway. More particularly, stably-transfected CHO cells growing in wells of micro-titer plates (96 well or 384 well) can be adapted to high through-put screening of libraries of compounds. The *CHOP* promoter is a preferable promoter due to its low basal activity. Libraries of test substances may be screened using this strategy. For example, the DIVERSET™ library of universally diverse, pre-designed 10,000-50,000 drug-like small molecules (ChemBridge Corporation, San Diego) may be used. Compounds in the library will be applied one at a time in an automated fashion to the wells of the microtitre dishes containing the transgenic cells described above. A compound that inhibits the activity of GADD34L and consequently activates the reporter driven by the CHOP promoter will be identified and this particular compound will be subjected to secondary testing in one or all of the assays described herein. The composition and the structure of the identified test substances will be determined by referring back to the ChemBridge database. The test substance may be developed into a therapeutic agent to prevent or treat a disease caused by oxidative stress.

The ELISA assay for measuring phosphorylated eIF2α, by means of commercially available antiserum, may be developed for high throughput screening. ELISA-type assays may be performed in microtitre plates. See, for example, Peraldi et

al., 1992, J. Biochem. 285: 71-78; Schraag, et al., 1993, Analytical Biochemistry 211: 233-239; Cleavland, 1990, Analytical Biochemistry 190: 249-253; Farley, 1992, Analytical Biochemistry 203: 151-157; and Lczaro, 1991, Analytical Biochemistry 192: 257-261. For evaluating the effects of a test substance on phosphorylation within the normal cellular context, one can also used the rapid and quantitative assays systems described in U.S. patent No. 5,763,198. For example, two embodiments may be contemplated as follows.

The extent of phosphorylation of a target protein may be measured by exposing cells that express the target protein to a test substance and, thereafter, lysing the cell to release the cellular contents. The target protein is isolated by incubating the cell lysate with a binding partner to a solid support and thereafter washing away non-bound cellular components. A detection procedure is performed to assess the presence or absence of phosphorylated residues on the protein as compared to lysates of control cells, which were not exposed to the test substance. Alternatively, the binding partner may be directed against the phosphorylated forms of the target protein, so that the steps of isolation and of detection of phosphorylation are performed simultaneously.

These assays offer several advantages. The exposure of the test substance to a whole cell allows for the evaluation of its activity in the natural context in which the test substance may act. In addition, radioactive labeling of the target cell proteins is not required in the assay. Because this assay can readily be performed in a microtitre plate format, the assays described can be performed by an automated robotic system, allowing for testing of large numbers of test samples within a reasonably short time frame.

An alternative embodiment of the invention relates to methods for determining the effect of a test substance on the ability of proteins, such as GADD34L, to dephosphorylate eIF2α in a cell-free system. To assess modulation of enzyme activity, the test substance is added to a reaction mixture containing the phosphatase complex and phosphorylated eIF2α bound to a solid support by an antibody. A detection procedure as described herein is performed on the substance to assess the presence or absence of the phosphorylated residues, and results are compared to those obtained for controls, i.e., reaction mixtures to which the test substance was not added.

The assays of the invention can be used as a screen to assess the activity of a previously untested compound or extract, in which case a single concentration is tested and compared to controls. These assays can also be used to assess the relative potency of a compound by testing a range of concentrations, in a range of 100 μM to 1 pM, for example, and computing the concentration at which the amount of phosphorylation is increased by one-half (IC50) compared to controls.

The whole cell assay of the invention described herein can be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay, such as a solid phase coated with a binding partner to a protein of interest, or a detection molecule. The cell-free assays of the invention may be performed, for example, by utilizing pre-packaged kits comprising any or all of the reagents of the assay.

High throughput screening example

CHO K1 cells may be obtained from ATCC and may be cultured in DMEM:F12 in the presence of 10% fetal calf serum (Atlantic Biological). A murine CHOP genomic fragment containing the CHOP promoter may be used. The fragment is 8.5 kb in length, wherein its 3' end corresponds to the Pm/I site in exon 3, nine nucleotides 5' to the initiation methionine of CHOP. CHO K1 cells may be transfected with expression vectors encoding GADD34L, or portions thereof (such as pCMV2FLAG FL GADD34L, pCMV2FLAG-ΔN GADD34L, or the retroviral clone CD-GSE) and with the CHOP genomic fragment linked to the GFP reporter gene by the Lipofectamine *plus* method (Gibco-BRL) using 1 μg plasmid DNA per 35 mm plate. Cells may plated 48 hours after transfection to form GADD34L/CHOP-GFP transient reporter cells. Alternatively, the transfection of the reporter plasmid may include 0.1 μg of the *Neo*^{r}-containing plasmid pCDNA3 (Invitrogen) followed by selection of transfected cells with 0.5 mg/ml of the aminoglycosidic antibiotic G418 (Fisher Scientific) for 10 days to establish stable clones containing the reporter.

GADD34L/CHOP-GFP reporter cells are plated into 96 well microtitre plates at $5X10^3$ cells per well. Individual compounds (test substances) from the DIVERSet™ library, a library of universally diverse, pre-designed 10,000-50,000 drug-like small molecules (ChemBridge Co.), may be tested. The test substances would be added one at

a time in an automated fashion at concentrations from 10^{-9} M to 10^{-6} M to the wells of the microtitre dishes containing the GADD34L/CHOP-GFP reporter cells. Test substances that inhibit GADD34L activity and consequently activate the CHOP gene are identified through fluorescence of the GFP reporter protein using FL600 Microplate Fluorescence and Absorbance Reader (Bio Tek).

Isolation and Characterization of GADD34L

Isolation of GADD34L as a genetic suppressor of the Integrated Stress Response

A new regulator of the phospho-eIF2α–dependent Integrated Stress Response (ISR) pathway was identified by screening a library of recombinant retroviruses for clones that inhibit the expression of a *CHOP::GFP* reporter. A forward somatic cell screen for Genetic Suppressor Elements (GSE) that inhibit ISR target gene expression in cultured Chinese Hamster Ovary (CHO) cells was used. 293T cells were co-transfected with a cDNA library and helper retroviral packaging genes to generate a pseudo-typed, replication defective retroviral library that was transduced into a CHO transgenic cell line containing a Green Fluorescent Protein (GFP) reporter controlled by the *CHOP* promoter (*CHOP::GFP* cell line).

To form the transgenic cell line, CHO-K1 cells were stably transformed with a *CHOP::GFP* reporter plasmid. The plasmid was constructed by fusing an 8.5-kb 5' murine *CHOP* gene fragment, whose 3' end is at the *Pm*lI site in exon 3, nine nucleotides 5' of the CHOP coding region, to enhanced green fluorescent protein (GFP) (CLONTECH Laboratories, Inc.) and termination sequences from the SV-40 virus [Wang, X.-Z., Harding, H.P., Zhang, Y., Jolicoeur, E.M., Kuroda, M., and Ron, D. (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J. 17:5708-5717]. A clone of *CHOP::GFP* cells was selected for low basal GFP activity and high inducibility by tunicamycin (Calbiochem-Novabiochem) and amino acid starvation, and was used in all subsequent studies. It is referred to as the parental line.

In wildtype cells, the ER stress-inducing drug tunicamycin activates the endogenous ISR target gene *CHOP* and its surrogate *CHOP::GFP* reporter. Cells that have acquired a GSE are predicted to have impaired *CHOP::GFP* expression. Following

retroviral transduction and tunicamycin treatment, cells with reduced *CHOP::GFP* signal were selected by fluorescence activated cell sorting (FACS). The pool of selected "GFP dull" cells was then transfected with plasmids encoding helper retroviral packaging genes in order to recover a pool of retroviruses enriched in putative GSEs. This enriched pool was transduced into the parental *CHOP::GFP* cell line and the process of virus rescue and cell sorting was repeated three times. Individual retroviral clones causing decreased *CHOP::GFP* reporter activity following tunicamycin stress were then analyzed.

Genomic DNA was prepared from these clonal cell lines, and the GSE cDNA inserts were recovered from integrated retroviral clones by PCR using the primers: 5'GAT CCA TGG ATG GAT GGC CAG3' (SEQ ID NO: 5) and 5'GCA TGC TTT GCA TAC TTC TGC CTG3' (SEQ ID NO: 6). Recovered cDNA inserts were ligated into the parental pre-retroviral plasmid, packaged into infectious particles, and transduced into parental *CHOP::GFP* CHO cells. Reconstitution of the defect in *CHOP::GFP* expression following tunicamycin treatment was taken as confirmation of the presence of a GSE in the retroviral clone.

This method allowed the isolation of a suppressor of the ISR in a retroviral clone named CD-GSE. The cDNA within this clone was sequenced using vector primers. The partial sequence of the CD-GSE hamster cDNA clone was found to be highly related to several mouse and human sequences deposited in Genbank. These sequences were related, but not identical, to the previously characterized protein GADD34. The protein encoded by the CD-GSE and Genbank clones is therefore hereinafter named GADD34-Like, or GADD34L. The CD-GSE clone cDNA encodes the COOH terminus of GADD34L that includes a domain predicted to bind to the catalytic subunit of PP1c (corresponding to amino acids 314-688 of the mouse protein or 320-705 of the larger human protein).

The full length human GADD34L cDNA (Figure 2) and translated amino acid (Figure 3) sequences are available from the National Center for Biotechnology Information (NCBI) website under Genbank Accession Number NM_032833. The full length mouse GADD34L cDNA (Figure 4) and amino acid (Figure 5) sequences were deduced following sequencing of IMAGE consortium (LLNL) cDNA clone 961134 (Genbank Accession Number AA547392), 3965646 (Genbank Accession Number

BE914017) and 3599662 (Genbank Accession Number BE380181) (all from Research Genetics , Hunstville AL) and from the published sequence of the following Genbank accession numbers BI654191, BB527493, BB214542, AA413655, AW910437, BB395632, BI663549, BE648121 and BG695164.

In order to isolate mouse or human GADD34L cDNA fragments suitable for sequencing, subcloning, and use in expression vectors, the desired cDNA fragment can be amplified from an appropriate RNA source using RT-PCR, where the primers are designed based on the provided full length cDNA sequences. For example, an approximately 2kb mouse GADD34L fragment can be isolated from mouse embryo fibroblast RNA via RT-PCR using the primers G34L.Hind5S (5'TG CCC AAG CTT CGG CGA TCG CAC GCC TGC TC3') (SEQ ID NO: 7) and G34L.Xho6AS (5'GAA ACT CTC GAG TAA GAG ACA GAG TGG GCA CG3') (SEQ ID NO: 8). This cDNA fragment comprises nucleotides 543 through 2603, inclusive, of the full length sequence, and encodes for amino acids 24-698 of the mouse GADD34L protein.

Characterization of GADD34L

(a) GADD34L is a negative regulator of the ISR

In order to study the function of GADD34L, the retroviral CD-GSE clone was used to drive expression of a partial GADD34L peptide in the parent *CHOP::GFP* CHO cell line. This expression of exogenous GADD34L from the retroviral clone CD-GSE attenuated CHOP induction and eIF2α phosphorylation in CHO cells following cell stress., indicating that the ISR was suppressed.

In one experiment, parental and CD-GSE-transduced *CHOP::GFP* CHO cells were treated for 8 hours with 1.75 μg/mL of tunicamycin or 50 μM of arsenite, or left untreated. Expression of the *CHOP::GFP* transgene was then assessed by FACS analysis to generate quantitative plots of GFP expression. While both tunicamycin and arsenite treatment substantially activated *CHOP::GFP* reporter expression in the parental CHO cells, neither treatment activated reporter expression in CHO cells expressing a partial GADD34L peptide from the CD-GSE retrovirus.

In a second experiment, parental and CD-GSE-transduced *CHOP::GFP* CHO cells were treated for varying amounts of time (0, 1, 2, or 4 hours, or 0, 15, 30, 45, or 60

minutes) with 1.75 μg/mL of tunicamycin or 50 μM of arsenite. Western blots of cell lysates were then probed with antisera specific for endogenous CHOP protein and phosphorylated eIF2α. As a protein loading control, the same blots were reacted with an antibody to eIF2α. The antisera for detecting total content of eIF2α and of eIF2α phosphorylated on serine 51 (P- eIF2α) have been described previously [Scorsone, K.A., Panniers, R., Rowlands, A.G., and Henshaw, E.C. (1987). Phosphorylation of eukaryotic initiation factor 2 during physiological stresses which affect protein synthesis. J. Biol. Chem 262:14538-14543; and DeGracia, D.J., Sullivan, J.M., Neumar, R.W., Alousi, S.S., Hikade, K.R., Pittman, J.E., White, B.C., Rafols, J.A., and Krause, G.S. (1997). Effect of brain ischemia and reperfusion on the localization of phosphorylated eukaryotic initiation factor 2 alpha. J. Cereb. Blood Flow Metab 17:1291-1302]. CHOP protein was detected by immunoblot as described previously [Wang, X.-Z., Lawson, B., Brewer, J., Zinszner, H., Sanjay, A., Mi, L., Boorstein, R., Kreibich, G., Hendershot, L., and Ron, D. (1996). Signals from the stressed endoplasmic reticulum induce C/EBP homologous protein (CHOP/GADD153). Mol. Cell. Biol. 16:4273-4280].

The control parental CHO cells showed strong induction of CHOP protein, and an increase in phosphorylated eIF2α levels, following tunicamycin or arsenite treatment. Conversely, GADD34L-expressing CHO cells failed to upregulate CHOP protein levels following tunicamycin or arsenite treatment. Similarly, GADD34L-expressing CHO cells showed severely decreased levels of P-eIF2α (despite strong expression of eIF2α) prior to tunicamycin or arsenite treatment, and furthermore failed to phosphorylate eIF2α following tunicamycin or arsenite treatment.

(b) GADD34L is part of an eIF2α-specific PP1c holophosphatase

The sequence similarity between GADD34L and GADD34 suggested that, like GADD34, GADD34L may function as a regulator of PP1c phosphatase. To test this hypothesis, the effect of GADD34L overexpression on the dephosphorylation of eIF2α, and the ability of GADD34L to associate with PP1c *in vivo*, was examined.

A first experiment revealed that cells overexpressing GADD34L have increased phosphatase activity directed toward eIF2α. In this assay, eIF2α from rabbit reticulocyte lysates was radiolabeled *in vitro* on serine 52 with purified PERK kinase and γ-^{32}P-ATP

as described previously [Harding, H., Zhang, Y., and Ron, D. (1999). Translation and protein folding are coupled by an endoplasmic reticulum resident kinase. Nature 397:271-274, and]. Radiolabeled P-eIF2α was then incubated in a dephosphorylation reaction as described in [Novoa, I.; Zeng, H., Harding, H., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol., 153, 1011-1022] for 30 min with lysates from CHO cells overexpressing or not overexpressing a partial GADD34L peptide from the CD-GSE retroviral clone. Levels of ^{32}P-eIF2α were then detected by autoradiography. While exposure to parental CHO cell lysate failed to cause substantial dephosphorylation of eIF2α, exposure to CHO lysate containing exogenous GADD34L resulted in near-complete dephosphorylation of eIF2α.

Another experiment showed that GADD34L and PP1c form a protein complex *in vivo*. For this assay, pCMV2Flag vector GADD34L expression constructs were transfected into 293T cells. The expression vector pCMV2FLAG FL GADD34L, containing the cDNA sequence encoding mouse GADD34L amino acids 24-698, was constructed by digesting the 2kb GADD34L RT-PCR product with HindIII and XhoI and ligating the fragment into HindIII and SalI digested pCMV2-Flag vector. The expression vector pCMV2FLAG-ΔN GADD34L, containing the cDNA sequence encoding mouse GADD34L amino acids 418-698, was constructed by ligating the NarI and XbaI fragment of IMAGE consortium clone 9611134 (Genbank Accession Number AA547392, corresponding to nucleotides 1711-2662 of the mouse GADD34 cDNA sequence in Figure 4A) into AccI and XbaI digested pCMV2-Flag vector. These expression vectors direct the expression of FLAG epitope tagged GADD43L fusion proteins in transfected cells.

Cultured transfected cells were lysed and the immune complex containing GADD34L was isolated by immunoprecipitation using the anti-FLAG antibody (Kodak, IBI). These immunoprecipitates were then immunoblotted. Recombinant GADD34L proteins on the immunoblot were detected with the anti-FLAG antibody, and co-immunoprecipitating endogenous PP1c detected with a rabbit anti–human PP1c antiserum (Santa Cruz Biotechnology, Inc.) at a dilution of 1:200.

PP1c protein was present in anti-FLAG immunoprecipitates containing FLAG-tagged FL GADD34L, and in those containing FLAG-tagged ΔN GADD34L, demonstrating that GADD34L forms a protein complex with PP1c *in vivo*. The ability of FLAG-ΔN GADD34L to associate with PP1c indicates that the domain responsible for this interaction is located in the C-terminal domain of the GADD34L protein, as predicted. Note that the transfected 293T cells were not subjected to stress prior to lysate immunoprecipitation. Thus, GADD34L association with PP1c is not dependent upon activation of the ISR.

(c) GADD34L is expressed constitutively in cells not subject to stress and is not regulated by stress

Further experimentation showed that GADD34L is expressed constitutively in cells not subject to stress and is not regulated by stress. Antiserum to mouse GADD34L can be raised by immunizing a rabbit with 5 injections of 0.3mg of bacterially expressed mouse GADD34L, where partial GADD34L peptides are expressed as fusion proteins from the pRSET A Vector (Invitrogen) or GST-fusions from the pGEX-2T vector^{Tm} (Pharmacia) in bacterial host cells. Thereafter, endogenous GADD34L protein was detected on Western blots of lysates of HT22 cells treated with tunicamycin (2μg/ml) and arsenite (50μM) for various time periods (0, 1, 2, or 4 hours).

GADD34 protein is present at low levels in cells not subject to stress, and its expression is dramatically upregulated following cell stress. Conversely, GADD34L was found constitutively in cells not subject to stress and its expression is not upregulated following cell stress. Given that endogenous GADD34L is constitutively expressed, and that association of GADD34L with PP1c is not dependent on activation of the IRS, it follows that the GADD34L:PP1c eIF2α holophosphatase is present constitutively in cells not subject to stress.

Inhibition of GADD34L activates the ISR in cells not subject to stress

The GADD34L:PP1c eIF2α holophosphatase complex may act to repress basal levels of eIF2α phosphorylation. Consequently, inhibition of GADD34L activity that abrogates function of the holophosphatase complex in cells not subject to stress may cause the build-up of phosphorylated eIF2α and activate the ISR. To test this hypothesis,

GADD34L activity was inhibited by two independent methods, using *GADD34L* mRNA directed RNA-interference (RNAi) and using the chemical compound 22P19 (see Figure 7). The methods described in these tests may be used in methods of screening to identify test substances which inhibit GADD34L.

RNAi-based inhibition of endogenous GADD34L activates the ISR in cells not subject to stress

The RNAi technique is based on the use of sequence-specific double stranded (complementary sense:antisense duplex) RNA to stimulate the degradation of target endogenous sense RNAs containing the same sequence. *GADD34L* mRNA targeted RNAi was used to inhibit the production of endogenous GADD34L in the parental *CHOP::GFP* CHO cell line. This inhibition activates the ISR as assayed by expression of the *CHOP::GFP* reporter.

Complementary double stranded RNA oligonucleotides of the sequence 5'-GGG AUG GAU GCA GGU UCC AdTdT-3' (SEQ ID NO: 9) and 5'-U GGA ACC UGC AUC CAU CCC dTdT-3' (SEQ ID NO: 10) were purchased from Dharmacon Inc. The oligonucleotides arrived in an annealed and desalted form from the manufacturer and were used to transfect CHO *CHOP::GFP* cells using Oligofectamine™ (Invitrogen, Life Science Inc). Briefly CHO cells were plated at 14000 cells per well of a 24-wells dish, grown overnight and transfected with 2.5μL of 20μM siRNA duplex using 1.5μL Oligofectamine in a total volume of 250μL of medium.

Cells were harvested 16, 24, or 44 hours after transfection. Expression of the *CHOP::GFP* transgene in these cells not subject to stress was then assessed quantitatively using by FACS analysis. By this method it was determined that inhibition of GADD34L using the siRNA duplex activates *CHOP::GFP* expression in cells not subject to stress.

Chemical compound 22P19 inhibits the eIF2α phosphatase activity of GADD34L and activates the ISR in cell not subject to stress

The chemical compound called 22P19 (see Figure 7) was isolated by screening the Chembridge™ library (Chembridge San Diego, CA) for compounds that protect PC-

12 cells from death induced by prolonged exposure to tunicamycin. Treatment of cells not subject to stress with 22P19 inhibits GADD34L phosphatase activity and activates the ISR.

The first experiment revealed that 22P19 inhibits the eIF2α phosphatase activity of cells overexpressing GADD34L. In this assay, eIF2α from rabbit reticulocyte lysates was radiolabeled *in vitro* on serine 52 with purified PERK kinase and γ-^{32}P-ATP as described previously [Harding, H., Zhang, Y., and Ron, D. (1999). Translation and protein folding are coupled by an endoplasmic reticulum resident kinase. Nature 397:271-274, and]. Radiolabeled P-eIF2α was then incubated for 0, 10, or 20 minutes in a dephosphorylation reaction with cell lysates [as described in Novoa, I.; Zeng, H., Harding, H., and Ron, D. (2001). Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2α. J. Cell Biol., 153, 1011-1022]. Cell lysates were from parental *CHOP::GFP* CHO cells and CD-GSE transformed *CHOP::GFP* CHO cells expressing exogenous GADD34L that were left untreated for 24 hours, treated with DMSO carrier for 24 hours, or treated with 70 μM 22P19 in DMSO for 4 or 24 hours prior to lysate preparation. Levels of ^{32}P-eIF2α were then detected by autoradiography. As expected, lysates from control GADD34L-expressing cells were able to substantially dephosphorylate eIF2α in a 20 minute dephosphorylation reaction (22 ^{32}P counts versus 100 input for untreated, and 13 ^{32}P counts versus 98 input for DMSO treated) Conversely, lysates from GADD34L-expressing cells that had been pretreated with 22P19 for 24 hours failed to substantially dephosphorylate eIF2α in a 20 minute dephosphorylation reaction (60 ^{32}P counts versus 99 input), similar to lysates from untreated parental cells not expressing exogenous GADD34L (65 ^{32}P counts versus 91 input). This result indicated that prolonged 22P19 exposure inhibited the phosphatase action of the exogenous GADD34L.

A second experiment showed that treatment with the GADD34L-inhibitor 22P19 activates the IRS in cells not subject to stress. In this assay, HT22 cells were treated with 70μM 22P19 for varying amounts of time (1, 4, 8, 16, 24, or 32 hours). Western blots of cell lysates were then probed with antisera specific for endogenous phosphorylated eIF2α (P-eIF2α), eIF2α, CHOP, and GADD34 proteins. Control untreated HT22 cells showed

low levels of P-eIF2α, despite strong eIF2α expression, contained no CHOP protein, and expressed very low levels of GADD34 protein. This protein profile represents the basal state of cells not subjected to stress. HT22 cells treated with 22P19 for 4 hours or more activated phosphorylation of eIF2α to generate P-eIF2α and activated expression of both CHOP and GADD34 proteins. This protein profile indicated that the ISR was activated in HT22 cells not subjected to stress by 22P19 treatment. Note that eIF2α phosphorylation and induction of ISR target genes was sustained in 22P19-treated cells for up to 32 hours. In cells subject to stress, eIF2α phosphorylation is typically transient, due to the induction of GADD34 which dephosphorylates eIF2α. But as 22P19 also inhibits GADD34 phosphatase activity, this rectifying response did not occur in 22P19-treated cells.

Preconditioning induced by inhibition of GADD34L protects cells against oxidative toxicity

The described experiments demonstrated that inhibition of GADD43L promotes an ISR in cells not subject to stress. In order to determine whether this preconditioning is protective against subsequent cell stress, cell survival rates of HT22 cells following toxic glutamate exposure were measured, where those HT22 cells had been preconditioned by 22P19- or RNAi- mediated inhibition of GADD34L (see Figure 6).

For this assay, HT22 cells were either treated with 70μM 22P19 for 24 or 48h, or transfected with GADD34L siRNA or control CD2 siRNA, and then exposed to 0mM, 5mM, or 10mM glutamate for 18 hours. Percent cell survival for each group was then calculated where 100% survival was defined as the MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Sigma) cleavage reduction in cells that had not been exposed to glutamate in each treatment group. Final cell survival calculations represented the means ± SEM of a representative experiment performed in duplicate and repeated four times. Percent survival of 22P19 and GADD34L siRNA preconditioned cells was then compared to that of non-preconditioned cells (Figure 6). Preconditioning with either 22P19 or GADD34L siRNA substantially improved cell survival following glutamate treatment, indicating that inhibition of GADD34L protects cells against oxidative stress.

This invention is thus directed to a method for screening a plurality of test substances useful for the prevention or treatment of a disease involving an oxidative stress, which comprises the steps of i) testing each of the test substances for its ability to inhibit the activity of GADD34L and ii) identifying the test substance which inhibits the activity of GADD34L, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. This invention is further directed to a method for identifying a test substance useful for the prevention or treatment of a disease involving an oxidative stress, which comprises testing a test substance for its ability to inhibit the activity of GADD34L, thereby to determine whether the substance promotes resistance to cell stress, and to identify said substance as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the test substance inhibits GADD34L activity by disrupting the formation of the GADD34L and PP1c protein complex. In another embodiment, the test substance inhibits the production of GADD34L protein from the GADD34L mRNA. In another embodiment, the test substance inhibits the production of GADD34L mRNA from the GADD34L genomic locus. In another embodiment, the method further comprises a step of verifying whether said test substance does not cause stress to cells.

The invention is directed to a method, which comprises the steps of i) contacting the test substance or each of the test substances with a cell-free composition containing GADD34L and PP1c proteins in the form of a purified complex and eIF2α in a phosphorylated form, ii) assessing the level of phosphorylation of eIF2α, in comparison with the level of phosphorylation determined in the absence of test substances, in a cell-free composition containing GADD34L and PP1c proteins in the form of a purified complex and eIF2α in a phosphorylated form, and iii) identifying the test substance which provides a higher level of phosphorylation of eIF2α, in comparison with the level of phosphorylation determined in the absence of test substance, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the assessment of the level of phosphorylation of eIF2α is effected by an immunoassay using an antibody that specifically recognizes the phosphorylated form of eIF2α. In another embodiment, the assessment of the level of

phosphorylation of eIF2α is effected by tracking the covalent binding of a radiolabelled phosphate group to eIF2α.

The invention is directed to a method, which comprises the steps of i) contacting a test substance or each of the test substances with cells not subject to stress that contain PP1c and eIF2α and overexpress GADD34L, or portions thereof, ii) assessing the level of phosphorylation of eIF2α after contact with the test substance or test substances, in comparison with the level of eIF2α phosphorylation in the absence of test substances, and iii) identifying the test substance which provides a higher level of phosphorylation of eIF2α, in comparison with the level of phosphorylation determined in the absence of test substance, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the assessment of the level of phosphorylation of eIF2α is effected by an immunoassay using an antibody that specifically recognizes the phosphorylated form of eIF2α. In another embodiment, the assessment of the level of phosphorylation of eIF2α is effected by tracking the covalent binding of a radiolabelled phosphate group to eIF2α. In an additional embodiment, the assessment of the level of phosphorylation of eIF2α is conducted additionally on exogenous radiolabeled eIF2α following either *in vivo* or *in vitro* exposure to cell lysates, where such assessment can provide more detailed information concerning the affect of test substances on the rate and enzyme kinetics of the eIF2α-specific phosphatase.

The invention is directed to a method, which comprises the steps of i) contacting a test substance or each of the test substances with cells that normally express endogenous GADD34L, ii) and identifying a test substance that inhibits the expression of endogenous GADD34L, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the level of GADD34L expression is assessed by determining the level of transcription of GADD34L. In a further embodiment, determination of the level of transcription of GADD34L is effected by means of a Northern blot. In a further embodiment determination of the level of transcription of GADD34L is effected by means of *in situ* hybridization. In another embodiment, the level of GADD34L expression is assessed by

the level of translation of GADD34L. In a further embodiment, determination of the level of translation of GADD34L is effected by means of an immunoassay.

The invention is directed to a method, which comprises the steps of i) contacting a test substance or each of the test substances with cells not subject to stress that overexpress GADD34L, or portions thereof, ii) assessing the expression of a target gene, and iii) identifying a test substance that activates the expression of the target gene, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the target gene is the CHOP gene.

The invention is directed to a method, which comprises the steps of i) obtaining cells not subject to stress that overexpress GADD34L, or portions thereof, and have been transfected with a reporter gene operatively associated with all or part of the promoter of a target gene, and ii) contacting a test substance or each of the test substances with these cells, and assaying the level of expression of said reporter gene, and iii) identifying a test substance that activates the expression of the reporter gene, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the target gene is the CHOP gene. In another embodiment, said reporter gene encodes one of the group consisting of GFP, CAT, GAL, LUC, and GUS.

The invention is directed to a method, which comprises the steps of i) obtaining cells not subject to stress which overexpress GADD34L, or portions thereof, ii) contacting a test substance or each of the test substances with the cells, in the presence of a toxic agent that induces oxidative stress, iii) quantitating cell survival of cells that overexpress GADD34L, or portions of GADD34L, following exposure to the toxic agent in the presence and absence of test substances, iv) and identifying a test substance that promotes cell survival of the cells following exposure to concentrations of toxic agent that induce oxidative stress, thereby to identify a test substance useful as a preventive or therapeutic agent for a disease involving an oxidative stress. In one embodiment, the toxic agent which induces oxidative stress is tunicamycin, arsenite, or glutamate.

In one embodiment, the identified test substance is useful for the prevention or treatment of a disease involving neuronal ischemia. In a further embodiment, the

identified test substance is useful for the prevention or treatment of a disease involving heart ischemia. In another embodiment, the identified test substance is useful for the prevention or treatment of renal damage induced by ischemia or toxins. In another embodiment, the identified test substance is useful for the prevention or treatment of an auto-immune disease. In a further embodiment, the selected compound is useful for the prevention or treatment of a neurodegenerative disorder.

The invention is directed to a method for the prevention or treatment of a disease involving an oxidative stress in a patient in need of such treatment, which comprises administering to the patient an effective amount of a GADD34L inhibitor identified for its ability to promote resistance to cell stress while not causing stress. In one embodiment, the disease is a disease involving neuronal ischemia, a disease involving heart ischemia, a disease involving renal damage induced by ischemia or toxins, an auto-immune disease, or a neurodegenerative disorder.

The invention is also directed to a pharmaceutical composition, comprising a therapeutic agent identified as being capable of promoting resistance to cell stress while not causing stress, in association with a pharmaceutically acceptable carrier. This therapeutic agent is defined as a substance that can activate the expression of a target gene of the Integrated Stress Response pathway, while not being toxic, which means more particularly that it does not cause stress to the cell. These properties may be determined by the methods of screening as above described.

Therapeutic agents that promote preconditioning and may be developed by this platform technology may more particularly prevent cognitive and neurological dysfunction in patients undergoing cardio-pulmonary bypass, if administered prophylactically or during the procedure. They may also protect the myocardium, kidneys and intestine from damage incurred during cardio-pulmonary bypass. Such therapeutic agents may also be efficacious in circumstances where neurological damage by ROS may be anticipated, such as early in the course of head trauma, in the post-neurosurgery period, following surgical procedures for brain re-vascularization, or in the treatment of status epilepticus.

The therapeutic agents may be administered locally to preserve function of specific organs that are to be subjected to ischemic stress. For example, intracoronary instillation to preserve myocardial function in preparation of cardiac surgery, or injection into the renal artery to preserve kidney function in preparation for surgery on the abdominal aorta. Therapeutic agents developed by this platform technology may be used ex-vivo in the preservation of organs and cells procured for purpose of transplantation from live or cadaver donors . For example, the essential role of PERK in promoting survival of pancreatic islets of Langerhans suggests that activating the ISR will promote survival of such cells ex-vivo and extend their utility in transplantation therapy of Diabetes Mellitus.

This platform technology may also be useful for identifying lead compounds for drug development to treat chronic diseases associated with cell and tissue damage caused by ROS. Examples of such conditions include Diabetes Mellitus, Parkinson's Disease and Cirrhosis.